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(54) Title: CANCER TREATMENT BY COMBINATION THERAPY

(57) Abstract: A method of treating cancer and compositions of use in such a method, the method including the step of administering, either sequentially or simultaneously, (i) a compound of the xanthenone acetic acid group of compounds, and (ii) at least one compound selected from compounds which modulate TNF production and compounds which act on biochemical pathways leading to TNF synthesis, the composition including a combination of (i) and (ii) above together with acceptable pharmaceutical carriers and/or vehicles.

CANCER TREATMENT BY COMBINATION THERAPY

FIELD OF THE INVENTION

5

This invention relates to a method of treating cancer and to compositions of use in such a method.

BACKGROUND OF THE INVENTION

10

The xanthenone acetic acid class of compounds have been shown to be of potential utility in cancer treatment. Of these, the compound 5,6-dimethylxanthenone-4-acetic acid (DMXAA) has been shown to have significant antitumour activity against murine tumours. Studies in animals have shown that this activity is a consequence of the induction of the cytokine tumour necrosis factor (TNF), particularly within tumour tissue, and of the consequent inhibition of tumour blood flow. To date, DMXAA has shown evidence of marginal clinical anti-cancer activity in humans.

The applicants have now surprisingly found that DMXAA amplifies the induction of TNF by cultured human peripheral blood cells in response to a variety of agents capable of inducing a second signal that by itself modulates TNF production. These include ligands that occupy external cellular receptors connected with the TNF induction pathway and compounds that modulate cellular biochemical pathways connected to TNF induction.

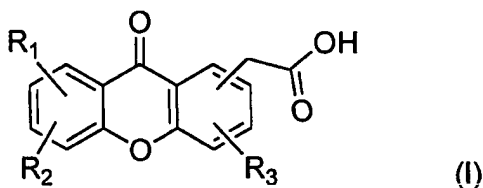
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With the above background in mind, it is an object of the present invention to provide a method of treatment of cancer which will at least provide the public with a useful choice.

30 **SUMMARY OF THE INVENTION**

Accordingly, in a first aspect, the present invention provides a method of treating cancer, the method including the step of administering to a mammal in need of such treatment, either simultaneously or sequentially:

- 5 (i) a compound of the formula (I)



or a pharmaceutically acceptable salt or ester thereof, wherein R_1 , R_2 and R_3 are each
 10 independently selected from the group consisting of H, C_1 - C_6 alkyl, halogen, CF_3 ,
 CN, NO_2 , NH_2 , OH, OR, $NHCOR$, $NHSO_2R$, SR, SO_2R or NHR , wherein each R is
 independently C_1 - C_6 alkyl optionally substituted with one or more substituents
 selected from hydroxy, amino and methoxy, and wherein each of R_1 , R_2 and R_3 may
 be present at any of the available positions 1 to 8;

15

and wherein in each of the carbocyclic aromatic rings in formula (I), up to two of the
 methine ($-CH=$) groups may be replaced by an aza ($-N=$) group;

and wherein any two of R_1 , R_2 and R_3 may additionally together represent the group
 20 $-CH=CH-CH=CH-$, such that this group, together with the carbon or nitrogen atoms
 to which it is attached, forms a fused 6 membered aromatic ring, and

(ii) a compound selected from compounds which modulate TNF production and
 compounds which act on biochemical pathways leading to TNF synthesis.

25

Preferably, the mammal is a human.

In certain preferred embodiments, the compound (ii) is a ligand that binds to the CD14 receptor of cells, such as bacterial LPS, deacylated LPS and CD14 receptor antibodies.

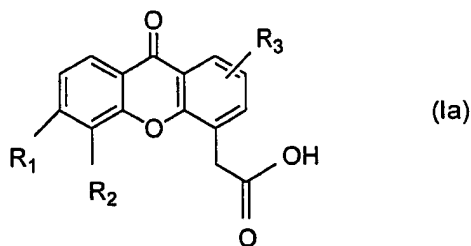
- 5 In other preferred embodiments, the compound (ii) is a ligand that binds to a surface receptor of cells connected with TNF production other than the CD14 receptor, such as interleukin-1 alpha.

10 In other preferred embodiments, the compound (ii) is a compound that induces protein kinase C, such as phorbol myristate ester.

In other preferred embodiments, the compound (ii) is a compound that can decrease the activity of protein phosphatases, preferably protein phosphatase 2A, such as okadaic acid.

15

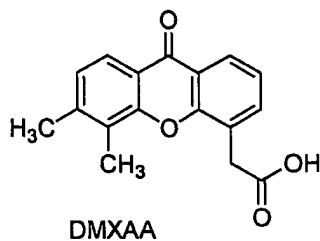
Preferably, compound (i) is of the formula (Ia):



wherein R₁, R₂ and R₃ are as defined for the compound of formula (I) above.

20

Most preferably, the compound of formula (I) or (Ia) is 5,6-dimethylxanthone-4-acetic acid, having the formula



In a further aspect, the present invention provides the use of a compound (i) of the formula (I) as defined above, or a pharmaceutically acceptable salt or ester thereof, in the manufacture of a medicament for treating cancer in a mammal by sequential or simultaneous co-administration of the medicament and a compound (ii) selected from compounds which modulate TNF production and compounds which act on biochemical pathways leading to TNF synthesis.

Preferably the compound (i) is DMXAA.

10 Preferably compound (ii) is selected from a ligand that binds to the CD14 receptor of cells; a ligand that binds to a surface receptor of cells connected with TNF production other than the CD14 receptor; a compound that induces protein kinase C; or a compound that can decrease the activity of protein phosphatases.

15 In still a further aspect, the present invention provides the use of a compound (ii) selected from compounds which modulate TNF production and compounds which act on biochemical pathways leading to TNF synthesis, in the manufacture of a medicament for treating cancer in a mammal by sequential or simultaneous co-administration of the medicament and a compound (i) of the formula (I) as defined above, or a pharmaceutically acceptable salt or ester thereof.

20 Preferably the compound (i) is DMXAA.

25 Preferably compound (ii) is selected from a ligand that binds to the CD14 receptor of cells; a ligand that binds to a surface receptor of cells connected with TNF production other than the CD14 receptor; a compound that induces protein kinase C; or a compound that can decrease the activity of protein phosphatases.

30 In yet a further aspect, the present invention provides a pharmaceutical composition suitable for treating cancer, the composition including a compound (i) of the formula (I) as defined above or a pharmaceutically acceptable salt or ester thereof, and a compound (ii) selected from compounds which modulate TNF production and

compounds which act on biochemical pathways leading to TNF synthesis, in combination with one or more pharmaceutically acceptable carriers or vehicles.

Preferably the compound (i) is DMXAA.

5

Preferably compound (ii) is selected from a ligand that binds to the CD14 receptor of cells; a ligand that binds to a surface receptor of cells connected with TNF production other than the CD14 receptor; a compound that induces protein kinase C; or a compound that can decrease the activity of protein phosphatases.

10

Preferably the composition is formulated for co-administration of compounds (i) and (ii), or is formulated for sequential administration of compounds (i) and (ii) in any order.

15 DESCRIPTION OF THE DRAWINGS

While the invention is broadly as defined above, it also includes embodiments of which the following description provides examples. These specific embodiments are described in conjunction with the accompanying drawings in which:

20

Figure 1 shows the effect of DMXAA on LPS-induced TNF production in HPBL *in vitro*. HPBL were incubated (8 h) with the indicated concentrations of LPS alone (no shading) or in combination with DMXAA (shading). Supernatants were then removed and assayed for TNF content;

25

Figure 2 shows the effect of DMXAA on dLPS-induced TNF production in HPBL *in vitro*. HPBL were incubated (8 h) with the indicated concentrations of dLPS alone (light bars) or in combination with DMXAA (shaded bars). Supernatants were then removed and assayed for TNF content. Horizontal lines represent the SEM;

30

Figure 3 shows the effect of anti-CD14 antibodies on DMXAA- and LPS-induced TNF production in HPBL *in vitro*. HPBL were incubated (8 h) with LPS (1 ng/ml or

1 $\mu\text{g/ml}$), DMXAA (800 $\mu\text{g/ml}$) or flavone acetic acid (FAA) (800 $\mu\text{g/ml}$) in the
absence (no shading) or the presence (shading) of anti-CD14 antibodies.
Supernatants were then removed and assayed for TNF content. Horizontal lines
represent the SEM;

5

Figure 4 shows the effect of DMXAA on TNF production in HPBL *in vitro* in
response to interleukin-1 α . HPBL were incubated (8 h) with the indicated
concentrations of drug either alone (filled symbols) or in combination with DMXAA
(unfilled symbols). Supernatants were then removed and assayed for TNF content.

10 Vertical lines represent the SEM;

Figure 5 shows the effect of DMXAA on TNF production in HPBL *in vitro* in
response to phorbol-12-myristate-13-acetate. HPBL were incubated (8 h) with the
indicated concentrations of drug either alone (unshaded) or in combination with
15 DMXAA (shaded). Supernatants were then removed and assayed for TNF content.
Horizontal lines represent the SEM; and

Figure 6 shows the effect of DMXAA on TNF production in HPBL *in vitro* in
response to okadaic acid. HPBL were incubated (8 h) with the indicated
20 concentrations of drug either alone (unshaded) or in combination with DMXAA
(shaded). Supernatants were then removed and assayed for TNF content. Horizontal
lines represent the SEM.

Figure 7 shows the effect of anti-CD14 antibodies and dLPS on TNF production in
25 response to LPS and DMXAA in murine leucocytes *in vitro*. Murine leucocytes
were pre-incubated for 15 minutes with or without either anti-CD14 antibodies (10
 $\mu\text{l/well}$) or dLPS (500 $\mu\text{g/ml}$) before the addition of DMXAA (800 $\mu\text{g/ml}$), DMXAA
(800 $\mu\text{g/ml}$) or LPS (1 ng/ml). Cultures were incubated for 8 hours and the TNF
content of the supernatant was measured.

30

Figure 8 shows the effect of antibiotic treatment on *in vivo* TNF production. Mice
were treated orally for three days with an antibiotic combination to reduce the

bacterial flora in the gut. Mice were then treated with DMXAA (25 mg/kg) and TNF was measured 24 hours later.

DESCRIPTION OF THE INVENTION

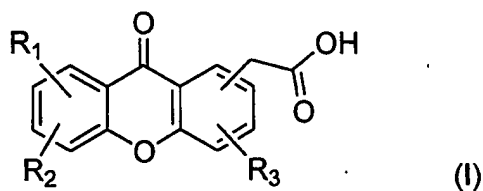
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As defined above, the present invention relates to a method of treating cancer and to compositions of use in such a method.

The invention resides in the applicant's unexpected finding of a very large synergistic interaction in cultured human peripheral blood cells between compounds of the xanthenone acetic acid class having the formula (I) as defined below and compounds capable of contributing to the control pathway that modulates TNF (tumour necrosis factor) synthesis in human cells, that is, compounds that themselves modulate TNF production or compounds which are capable of acting on pathways leading to TNF synthesis.

15

FORMULA (I)



20

or a pharmaceutically acceptable salt or ester thereof, wherein R_1 , R_2 and R_3 are each independently selected from the group consisting of H, C_1 - C_6 alkyl, halogen, CF_3 , CN, NO_2 , NH_2 , OH, OR, $NHCOR$, $NHSO_2R$, SR, SO_2R or NHR , wherein each R is independently C_1 - C_6 alkyl optionally substituted with one or more substituents selected from hydroxy, amino and methoxy, and wherein each of R_1 , R_2 and R_3 may be present at any of the available positions 1 to 8;

25

and wherein in each of the carbocyclic aromatic rings in formula (I), up to two of the methine ($-CH=$) groups may be replaced by an aza ($-N=$) group;

and wherein any two of R₁, R₂ and R₃ may additionally together represent the group -CH=CH-CH=CH-, such that this group, together with the carbon or nitrogen atoms to which it is attached, forms a fused 6 membered aromatic ring.

5

In particular, the simultaneous administration of a compound of formula (I) 5,6-dimethylxanthenone-4-acetic acid (DMXAA) and a compound capable of contributing to the control pathway that modulates TNF synthesis in human cells, shows greater induction of TNF in cultured human peripheral blood cells than either agent alone. TNF has recognised anticancer activity and can act either directly on cancer cells or indirectly on the cancer's blood supply.

It is shown herein that while DMXAA alone has little effect on TNF induction in cultured human peripheral blood leucocytes (HPBL), its combination with compounds that contribute to TNF induction surprisingly achieves effects dramatically larger than for either agent alone, and greatly exceeds the sum of effects of the individual agents. The combination of DMXAA or other compounds of the formula (I) (as described above) with a second agent acting on the TNF pathway is therefore expected to have clinical utility in cancer treatment. Further studies are needed to confirm that all compounds of formula (I) will act in a similar manner to DMXAA, but at this stage there is little reason to presume that DMXAA will be alone amongst the xanthenone acetic acid compounds in its effect in combination with the compounds (ii).

The compounds of the formula (I) are known and can be prepared using methods known to those persons skilled in the art. For example, compounds of the formula (I) and their preparation are described in the following references:

- Journal of Medicinal Chemistry* 34(1): 217-22, January 1991;
30 *Journal of Medicinal Chemistry* 34(2): 491-6, February 1991;
Journal of Medicinal Chemistry 33(5): 1375-9, May 1990;
Journal of Medicinal Chemistry 34(9): 2864-70, September 1991; and

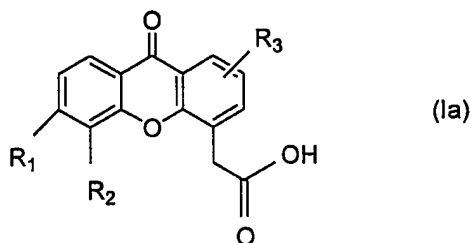
Journal of Medicinal Chemistry 32(4): 793-9, April 1989,

the contents of which are incorporated herein by reference.

- 5 Of the compounds of formula (I) defined above, compounds of the formula (Ia) as described below (in which the substituents R_1 and R_2 are at the 5- and 6-positions), are generally preferred for use in the methods of the invention.

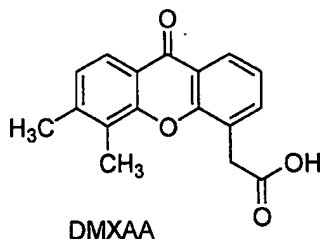
FORMULA (Ia)

10



wherein R_1 , R_2 and R_3 are as defined for the compound of (I) above.

- 15 A particularly preferred compound is 5,6-dimethylxanthone-4-acetic acid (DMXAA). The preparation of this compound is described in *Journal of Medicinal Chemistry* 34(1): 217-22, January 1991.



- 20 The compounds capable of contributing to the control pathway that modulates TNF synthesis in human cancer tissue described above are also well known compounds (see, for example, Philpott M, Ching LM, Baguley BC; *Eur J Cancer* 2001, in press) and can likewise be prepared by methods known to those skilled in the art. As will be readily apparent, more than one of those compounds can be combined with the

compound(s) of formula (I) or (Ia). Reference to "a compound" should not be seen to be restrictive to only one such compound.

- 5 In certain embodiments of the invention, the compound capable of contributing to the control pathway that modulates TNF synthesis is a ligand that binds to the CD14 receptor of cells. Examples of such ligands are bacterial lipopolysaccharide (LPS), deacylated lipopolysaccharide (dLPS), and antibodies to the CD14 receptor for LPS and dLPS.
- 10 In further embodiments of the invention, the compound capable of contributing to the control pathway that modulates TNF synthesis is a compound that acts on surface receptors, other than CD14 receptors, that are connected with TNF production. An example of such a compound is interleukin-1 alpha (IL-1).
- 15 In still further embodiments of the invention, the compound is capable of contributing to the control pathways that modulate TNF synthesis by inducing the enzyme protein kinase C. Examples of such compounds are phorbol myristate esters such as phorbol myristate acetate.
- 20 In still further embodiments of the invention, the compound is capable of decreasing the activity of protein phosphatases, preferably protein phosphatase 2A. An example of such a compound is okadaic acid.
- 25 The therapeutic methods of the present invention therefore include the step of administering to a patient, simultaneously or sequentially, an agent capable of contributing to the control pathway that modulates TNF synthesis, and a compound of the formula (I) as defined above or a pharmaceutically acceptable salt or ester thereof.
- 30 The compound of formula (I) and the compound capable of contributing to the control pathway that modulates TNF synthesis can be administered to a patient in any suitable form. For example, the compounds may conveniently be administered

intravenously, using formulations for each compound already known in the art. The formulation of medicaments for use in cancer treatment utilising combinations of the compounds referred to herein, together with pharmaceutically acceptable carriers, vehicles and excipients would be well within the abilities of a person skilled in this art. One known precaution would be to protect solutions of the highly water soluble DMXAA compound from light.

The compounds of formula (I) and the compound capable of contributing to the control pathway that modulates TNF synthesis can be administered either simultaneously or sequentially, i.e. the compound capable of contributing to the control pathway that modulates TNF synthesis can be administered either before or after the compound of formula (I) is administered. Simultaneous co-administration, in most cases, is likely to be preferred.

The invention will now be described in more detail with reference to the following non-limiting examples. While the examples have been directed to specific combinations of compounds it will be appreciated by those skilled in this art that the results are not restrictive to those compound combinations only.

EXAMPLES

Methods

Incubation of HPBL with drugs

Partially purified buffy coats were purchased from Auckland Blood Centre and divided into 15-ml aliquots in 50-ml centrifuge tubes (2070 Conical Tubes, Becton Dickinson Labware, New Jersey, USA). HPBL in tissue culture dishes (10 ml; 10^7 cells/ml) were incubated overnight in α -MEM culture medium supplemented with FCS (10% v/v), streptomycin sulphate (100 μ g/ml) and penicillin-G (100 units/ml). All extraction operations were carried out at 7°C to prevent clotting. Unsupplemented α -MEM medium was added to 30 ml and a 10-ml layer of Ficoll-Paque PLUS was slowly added to the bottom of the tubes. After centrifugation at

300 g for 30 min the upper layer was removed and the HPBL layer was carefully drawn off into a fresh 50-ml centrifuge tube. The volume was adjusted to 50 ml, the cells were centrifuged at 300 g, and HPBL were resuspended in supplemented α -MEM medium and added (1 ml/well) to 24 well plates (Nunc, Kamstrup, Roskilde, Denmark). Agents (made up at twice the final concentration) were added and plates were incubated for the appropriate times in 5% CO₂/air at 37°C overnight. DMXAA sodium salt (this laboratory) was dissolved in medium and protected from light. FAA (National Cancer Institute, USA) was dissolved in 5% (w/v) sodium bicarbonate and diluted with medium. Interleukin-1 α (R&D Systems, USA), okadaic acid, LPS and deacylated LPS (Sigma Chemical Co., USA) were dissolved in α -MEM, filter-sterilised and used immediately. The MEM-18 mouse anti-human CD14 IgG antibody was obtained from Sanbio bv, am Uden, Netherlands, and was freed from azide before use by ultrafiltration.

15 *Measurement of TNF*

After the appropriate incubation period of HPBL with drug, supernatants were either used immediately or stored at -20°C. TNF standards were prepared by making serial dilutions of the TNF stock solution in supplemented culture media (concentration range 10 – 10,000 pg/ml). ELISA plates were made using the OptEIA Human TNF-alpha Set (Pharmingen, San Diego, CA, USA). TNF standards and samples were added to the ELISA plates and the assays were carried out according to the makers' directions.

25 *Example 1*

The induction of TNF in peripheral blood monocytes by low concentrations of the bacterial cell wall lipopolysaccharide (LPS) was unexpectedly stimulated by DMXAA. LPS has a large range of biological effects including antitumor effects (Raetz CRH, Ulevitch RJ, Wright SD, Sibley CH, Ding AH, Nathan CF. *FASEB J* 1991, 5, 2652-2660). Certain bacteria can localise in tumour tissue (Kimura NT, Taniguchi S, Aoki K, Baba T, *Cancer Res.* 1980, 40, 2061-2068) and would

therefore provide a localised LPS signal. Co-administration of DMXAA would amplify this signal.

Example 2

5

The induction of TNF by low local concentrations of the modified bacterial cell wall components, which by themselves do not stimulate TNF production, may be stimulated by DMXAA. Such components are included in genetically modified bacteria that might localise in tumour tissue but produce an attenuated systemic response, thus eliminating endotoxic shock as a side effect of such therapy (Low KB, Ittensohn M, Le T, Platt J, Sodi S, Amoss M, Ash O, Carmichael E, Chakraborty A, Fischer J, Lin SL, Luo X, Miller SI, Zheng LM, King I, Pawelek JM, Bermudes D, Nature Biotechnology, 1999, 17, 37-41.

15 The induction of TNF in peripheral blood monocytes by deacylated LPS (dLPS), an inactive form of LPS, was unexpectedly stimulated by DMXAA. dLPS does not alone induce TNF, and competitively inhibits the induction of TNF by LPS by competition for the CD14 receptor (Riedo FX, Munford RS, Campbell WB, Reisch JS, Chien KR, Gerard RD. *J Immunol* 1990, 144, 3506-3512). dLPS (500 µg/ml; 15 minutes pre-incubation) only slightly induced TNF production above the controls. dLPS also strongly reduced TNF production in response to LPS (1 ng/ml). DMXAA alone (800 µg/ml) caused no substantial induction of TNF. However the combination of dLPS (500 µg/ml; 15 minutes pre-incubation) and DMXAA (800 µg/ml) caused a large increase in TNF production.

25

Example 3

The induction of TNF in peripheral blood monocytes by an antibody (MEM-18) to the LPS receptor, CD14, was unexpectedly stimulated by DMXAA. Anti-CD14 antibody does not alone induce TNF alone and inhibits the induction of TNF by LPS (Devitt A, Moffatt OD, Raykundalia C, Capra JD, Simmons DL, Gregory CD, Nature 1998, 392, 505-509).

30

Example 4

The induction of TNF in peripheral blood monocytes by cytokines such as interleukin-1 (IL-1) was unexpectedly stimulated by DMXAA. The cytokine IL-1 is an inflammatory cytokine that itself has been reported to have experimental antitumor activity (Braunschweiger PG, Johnson CS, Kumar N, Ord V, Furmanski P, Cancer Res. 1988 48, 6011-6016). As shown in Figure 4, IL-1 alone is capable of inducing TNF in human peripheral blood leukocytes (HPBL). However, co-administration of DMXAA greatly increases (up to 56-fold in this case) the induction of TNF as compared to that by IL-1 alone.

Example 5

The induction of TNF by low molecular weight activators of protein kinase C such as phorbol myristate acetate (PMA) was unexpectedly enhanced by co-administration of DMXAA. When HPBL were incubated with PMA alone at concentrations up to 20 ng/ml, there was no substantial induction of TNF. DMXAA alone (800 µg/ml) also had no substantial effect, DMXAA but in combination with PMA induced a higher degree of TNF production. At concentrations higher than 20 ng/ml, PMA alone induced TNF synthesis, as has been reported by others (Dong ZY, Lu S, Zhang YH. *Immunobiol* 1989, 179, 382-394).

Example 6

The induction of TNF by low molecular weight protein phosphatase inhibitors such as okadaic acid (OA), was unexpectedly enhanced by co-administration of DMXAA. When HPBL were incubated with OA alone at concentrations up to 20 ng/ml, there was no substantial induction of TNF. DMXAA alone (800 µg/ml) also had no substantial effect, DMXAA but in combination with OA induced a higher degree of TNF production. At concentrations higher than 20 ng/ml, OA alone induced TNF

synthesis, as has been reported by others (Sung SSJ, Walters JA, Fu SM, J. Exp. Med. 1992, 176, 897-901).

Example 7

5

The effect of DMXAA on cultured murine leucocytes has also been investigated using LPS as a control. TNF was measured by enzyme-linked immunosorbent assay after 8 h.

Materials

- 10 DMXAA sodium salt (this laboratory) was dissolved in medium and protected from light (9). LPS and deacylated LPS (Sigma Chemical Co., MO) were dissolved in α -MEM, filter-sterilised and used immediately. The MEM-18 mouse anti-human CD14 IgG antibody was obtained from Sanbio bv, am Uden, Netherlands, and was freed from azide before use by ultrafiltration and was LPS-free (Endospecy ES-50M LPS
15 quantitation system, Seikagaku Corporation, Tokyo, Japan).

Extraction of murine leucocytes

- Blood samples for extraction of leucocytes were taken by cardiac puncture of halothane-anaesthetised mice into 1-ml syringes containing ACD-A anticoagulant (0.1 ml). All extraction operations were carried out at 7°C to prevent clotting.
- 20 Samples were pooled and unsupplemented α -MEM was added to 30 ml and a 10-ml layer of Ficoll-Paque PLUS was slowly added to the bottom of the tubes. After centrifugation at 300×g for 30 min the upper layer was removed and the leucocyte layer was carefully drawn off into a fresh 50-ml centrifuge tube. The volume was adjusted to 50 ml with unsupplemented growth medium, the cells were centrifuged at
25 300×g, and the leucocytes were resuspended at 10^7 cells/ml in α -MEM supplemented with foetal bovine serum (10% v/v), streptomycin sulphate (100 µg/ml) and penicillin-G (100 units/ml).

In vitro studies with murine leucocytes

Cells were added either to 24 well plates (1 ml/well; Nunc, Kamstrup, Roskilde, Denmark) or to 100 mm Petri dishes (10 ml/plate) and incubated in 5% CO₂/air at 37°C overnight. Agents (made up at twice the final concentration in growth medium) were added and plates were further incubated for 8 hours. After the appropriate incubation period of HPBL with drug in 24-well plates, supernatants were removed and either assayed immediately or stored at -20°C.

As seen in Figure 7, control cells, cells treated with anti-CD14 antibodies alone, or cells treated with dLPS alone produced very low concentrations of TNF. LPS significantly increased TNF production and this increase was abolished by co-incubation with anti-CD14 antibody or dLPS. DMXAA alone did not significantly increase TNF production, but co-incubation with anti-CD14 antibody resulted in a high level of TNF production that was even greater than that caused by LPS. Co-incubation with dLPS also caused a significant ($p < 0.001$) elevation of TNF production, although the magnitude of the effect was less than that caused by anti-CD14 antibody.

Example 8

The possible role of LPS for the *in vivo* production of TNF in mice was reviewed by pre-treating mice with a combination of antibiotics designed to sterilise the gut. The results support the concept that DMXAA acts as a co-stimulator with other inducers of TNF in both murine and human mononuclear cells.

To test the hypothesis that low concentrations of LPS synergise with DMXAA for *in vivo* TNF production, mice were treated orally with antibiotics for 3 days and then treated with 25 mg/kg DMXAA. TNF levels were measured 24 hours later.

In vivo studies

C57BL mice were either untreated, or treated for 4 days prior to DMXAA with a mixture of antibiotics (cephalocin 2 g/l and neomycin 2 g/l in the drinking water). Mice received a single i.p. dose of DMXAA (25 mg/kg) and blood was collected by

cardiac puncture of halothane-anaesthetised mice after 3 hours. Blood from each mouse was transferred to individual microcentrifuge tubes and allowed to clot overnight on ice before centrifugation at 2000×g for 20 minutes at 4°C. If clotting was not complete the sample was allowed to stand on ice for a further 2 hours, after
5 which it was re-centrifuged. Serum was drawn off the top of the blood samples and stored at -20°C until assay of TNF content.

Measurement of TNF

TNF standards were prepared by making serial dilutions of the TNF stock solution in supplemented culture media (concentration range 10 – 10,000 pg/ml). ELISA
10 (enzyme-linked immunosorbent assay) plates were made using the OptEIA Human TNF-alpha-Set (Pharmingen, San Diego, CA, USA). TNF standards and samples were added to the ELISA plates and the assays were carried out according to the manufacturer's directions.

15

Effect of Antibiotic Treatment

TNF concentrations were low in control mice and in mice treated with antibiotics alone. As shown in Figure 8, treatment with DMXAA substantially increased serum
20 TNF concentrations in mice not receiving antibiotics. Administration of DMXAA to mice following antibiotic treatment also increased serum TNF but the increase was significantly smaller ($p < 0.001$) than that in mice not receiving antibiotic treatment.

Discussion

25

The results of the above Examples (1-8) show that DMXAA appears to require a second signal for the induction of TNF synthesis with human leucocytes, and also demonstrates a similar effect with murine leucocytes. It is notable and unexpected that anti-CD14 antibody and dLPS, which bind to the CD14 receptor for LPS and
30 thus inhibit TNF induction by LPS, provide a signal that enables DMXAA to induce TNF (Fig. 6). The results of antibiotic pre-treatment (Fig. 7) strongly support the

hypothesis that small amounts of circulating bacterial products, which could include LPS or LPS products, are required for the TNF response to DMXAA.

5 The results suggest that combination of DMXAA with a strategy for increasing the second signal in tumour tissue, such as by use of compounds capable of contributing to modulation of TNF synthesis, may lead to a combination therapy having significant clinical anti-tumour effect. The increased induction of TNF, and the resultant effect this will have on tumour growth, is a significant advance of considerable public interest.

10

INDUSTRIAL APPLICATION

As will be apparent from the above description and examples, the present invention provides an improved method of cancer therapy that is expected to find widespread clinical utility. The invention also provides compositions of use in such methods of cancer therapy.

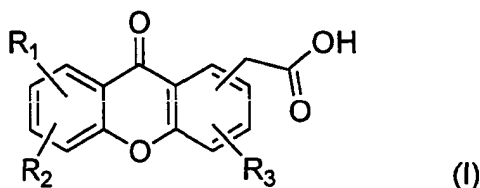
Those persons skilled in the art will understand that the specific description provided thereof is exemplary only and that the present invention is not limited thereto.

20 Alterations and modifications that would be apparent to a person skilled in the art are intended to be included within the spirit and scope of the invention as defined in the appended claims.

CLAIMS

1. A method of treating cancer, the method including the step of administering to a mammal in need of such treatment, either simultaneously or sequentially:

(i) a compound of the formula (I)



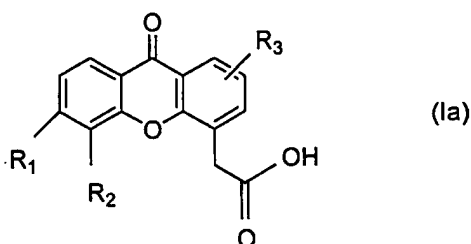
or a pharmaceutically acceptable salt or ester thereof, wherein R_1 , R_2 and R_3 are each independently selected from the group consisting of H, C_1 - C_6 alkyl, halogen, CF_3 , CN, NO_2 , NH_2 , OH, OR, $NHCOR$, $NHSO_2R$, SR, SO_2R or NHR , wherein each R is independently C_1 - C_6 alkyl optionally substituted with one or more substituents selected from hydroxy, amino and methoxy, and wherein each of R_1 , R_2 and R_3 may be present at any of the available positions 1 to 8; wherein in each of the carbocyclic aromatic rings in formula (I), up to two of the methine ($-CH=$) groups may be replaced by an aza ($-N=$) group; wherein any two of R_1 , R_2 and R_3 may additionally together represent the group $-CH=CH-CH=CH-$, such that this group, together with the carbon or nitrogen atoms to which it is attached, forms a fused 6 membered aromatic ring, and

(ii) a compound selected from compounds which modulate TNF production and compounds which act on biochemical pathways leading to TNF synthesis.

2. The method according to claim 1 wherein the compound (ii) is selected from a ligand that binds to the CD14 receptor of cells; a ligand that binds to

a surface receptor of cells connected with TNF production other than the CD14 receptor; a compound that induces protein kinase C; and a compound that can decrease the activity of protein phosphatases.

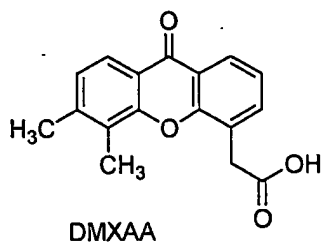
- 5 3. The method of claim 1 or claim 2 wherein compound (ii) is selected from bacterial LPS, deacylated LPS, CD14 receptor antibodies, interleukin-1 alpha, phorbol myristate ester and okadaic acid.
4. The method of any one of the previous claims wherein compound (i) is a
10 compound of the formula:



wherein R₁, R₂ and R₃ are as defined in claim 1.

15

5. The method of claim 1 or claim 4 wherein compound (i) is 5,6-dimethylxanthone-4-acetic acid, having the formula



- 20 6. The use of a compound (i) of the formula (I) as defined in claim 1, or a pharmaceutically acceptable salt or ester thereof, in the manufacture of a medicament for treating cancer in a mammal by sequential or simultaneous co-administration of the medicament and a compound (ii) selected from

compounds which modulate TNF production and compounds which act on biochemical pathways leading to TNF synthesis.

- 5
7. The use according to claim 6 wherein the compound (i) is DMXAA.
8. The use according to claim 6 or claim 7 wherein compound (ii) is a compound selected from at least one of the following: a ligand that binds to the CD14 receptor of cells; a ligand that binds to a surface receptor of cells connected with TNF production other than the CD14 receptor; a compound that induces protein kinase C; or a compound that can decrease the activity of protein phosphatases.
- 10
9. The use of a compound selected from compounds which modulate TNF production and compounds which act on biochemical pathways leading to TNF synthesis, in the manufacture of a medicament for treating cancer in a mammal by sequential or simultaneous co-administration of the medicament and a compound (i) of the formula (I) as defined above, or a pharmaceutically acceptable salt or ester thereof.
- 15
10. The use according to claim 9 wherein compound (i) is DMXAA.
- 20
11. The use according to claim 9 or claim 10 wherein compound (ii) is a compound selected from at least one of the following: a ligand that binds to the CD14 receptor of cells; a ligand that binds to a surface receptor of cells connected with TNF production other than the CD14 receptor; a compound that induces protein kinase C; or a compound that can decrease the activity of protein phosphatases.
- 25
12. A pharmaceutical composition suitable for treating cancer, including a compound (i) of the formula (I) as defined in claim 1 or a pharmaceutically acceptable salt or ester thereof, and a compound (ii) selected from compounds which modulate TNF production and compounds which act on
- 30

biochemical pathways leading to TNF synthesis, in combination with one or more pharmaceutically acceptable carriers or vehicles.

13. The composition according to claim 12 wherein compound (i) is DMXAA.
14. The composition according to claim 12 or claim 13 wherein compound (ii) is a compound selected from at least one of the following: a ligand that binds to the CD14 receptor of cells; a ligand that binds to a surface receptor of cells connected with TNF production other than the CD14 receptor; a compound that induces protein kinase C; or a compound that can decrease the activity of protein phosphatases.
15. A composition method or use, combining a compound of formula (I) as defined in claim 1 and a compound (ii) as defined in claim 1 substantially as herein described with reference to any one of the examples.

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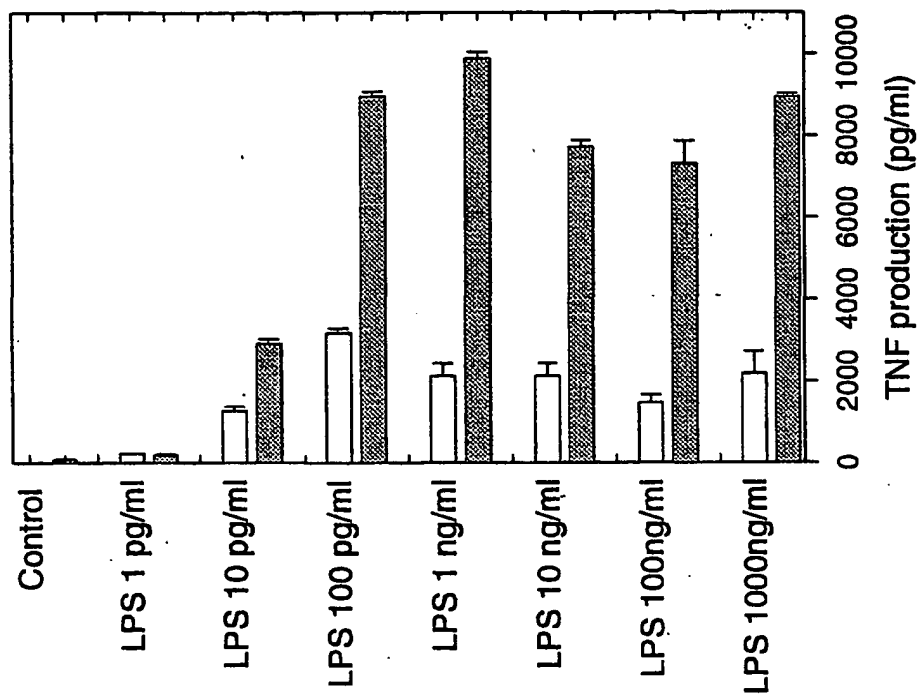


FIGURE 1

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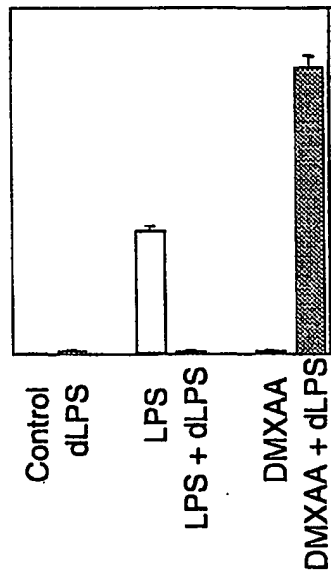


FIGURE 2

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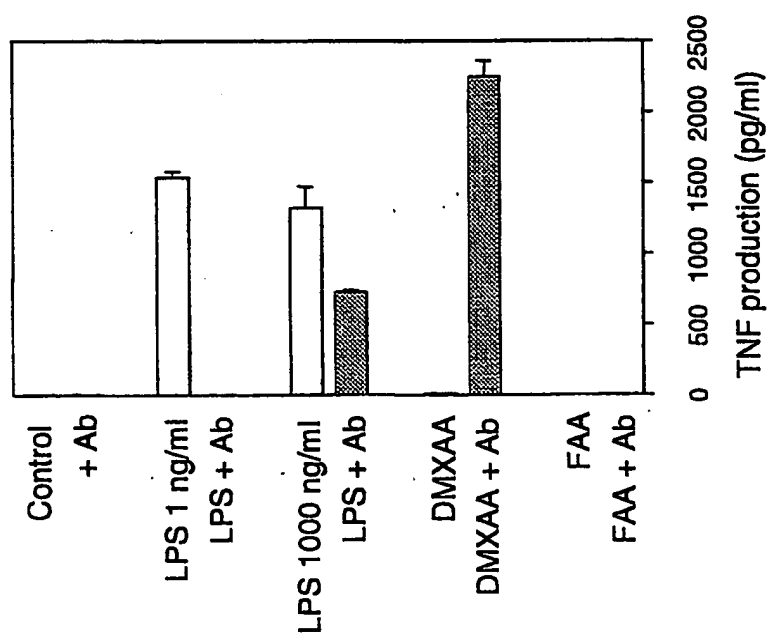


FIGURE 3

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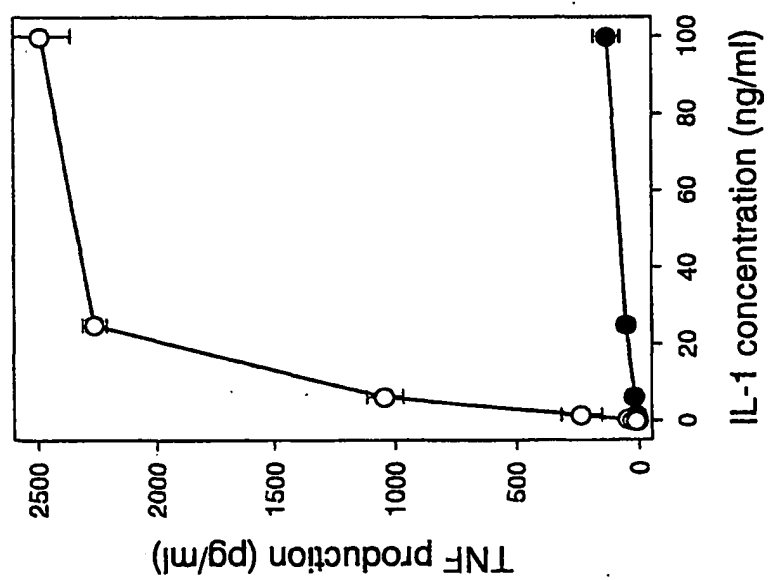


FIGURE 4

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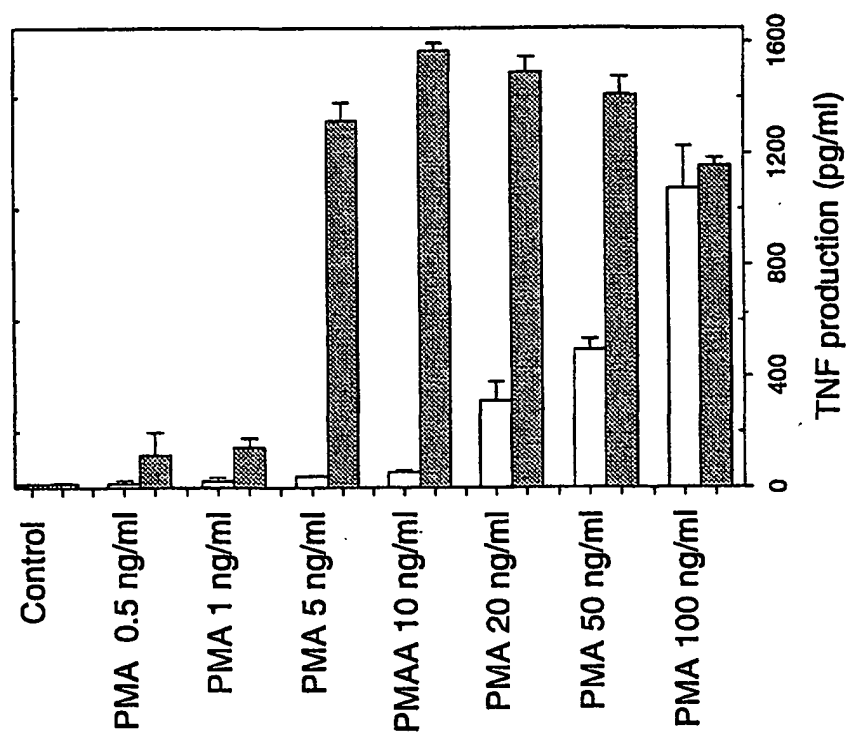


FIGURE 5

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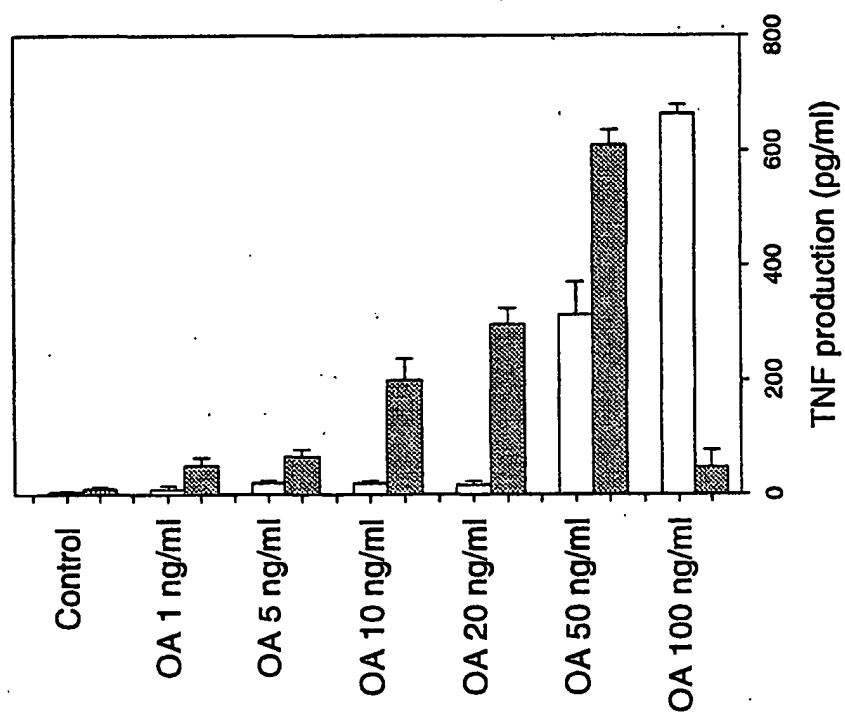


FIGURE 6

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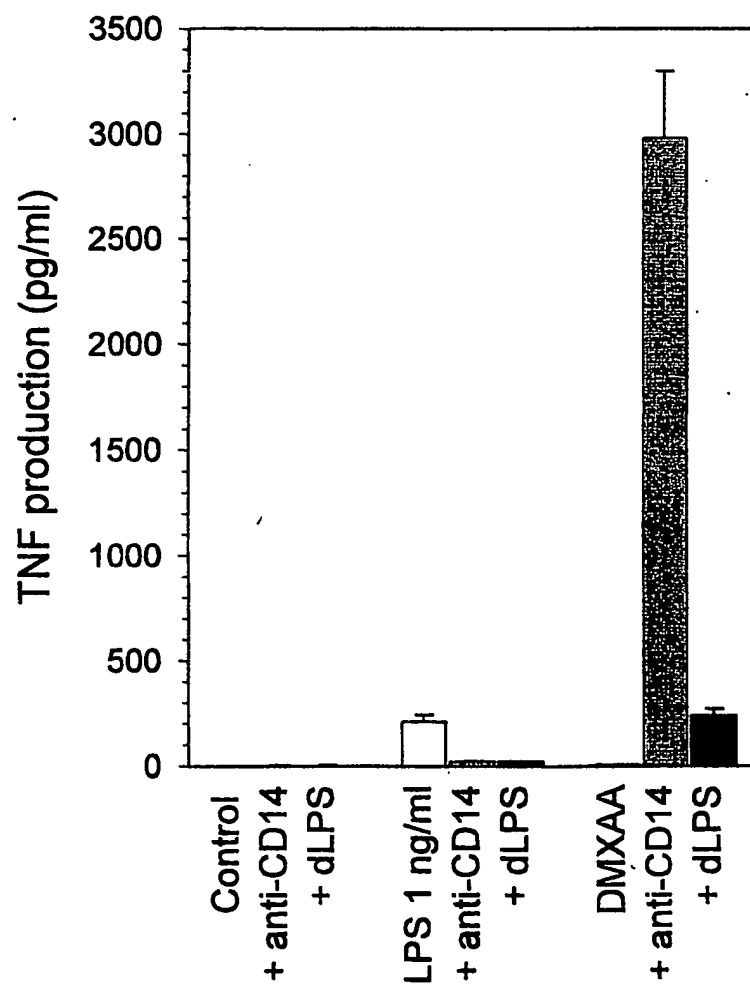


FIGURE 7

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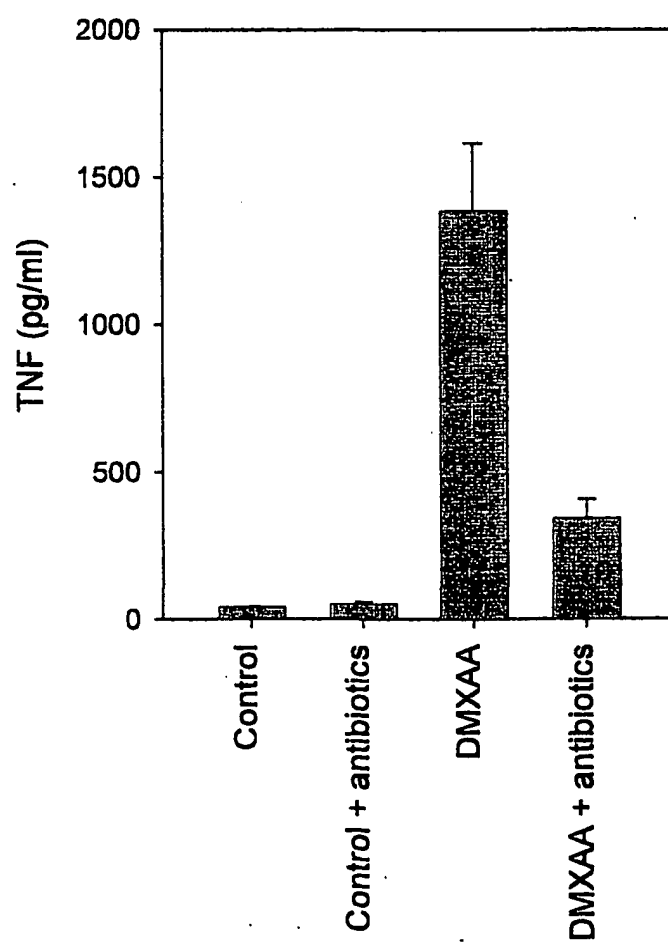


FIGURE 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ01/00154

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl⁷: A61K 31/352, A61P 35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: A61K 31/352, A61P 35/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

AU: IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DERWENT: Xanthenone, tumour, TNF, CD14, protein kinase, phosphatase, and related terms

MEDLINE: As above

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|-----------------------|
| X | CHING LM et al. , Cancer Chemotherapy and Pharmacology, 1994, 35 (2), "Interaction between endotoxin and the antitumour agent 5, 6-dimethylxanthenone-4-acetic acid in the induction of tumour necrosis factor and haemorrhagic necrosis of colon 38 tumours", pages 153-160 Whole document | 1-15 |
| A | FUTAMI H et al, Journal of Immunotherapy, Nov. 1992, 12 (4), "Cytokine induction and therapeutic synergy with interleukin-2 against murine renal and colon cancers by xanthenone-4-acetic acid derivatives", pages 247-255 Whole document | 1-15 |

☒ Further documents are listed in the continuation of Box C

☐ See patent family annex

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|---|--|
| * Special categories of cited documents: | "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" Document defining the general state of the art which is not considered to be of particular relevance | "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" earlier application or patent but published on or after the international filing date | "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
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| "O" document referring to an oral disclosure, use, exhibition or other means | |
| "P" document published prior to the international filing date but later than the priority date claimed | |

Date of the actual completion of the international search

19 October 2001

Date of mailing of the international search report

30 OCT 2001

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INTERNATIONAL SEARCH REPORT

national application No.

PCT/NZ01/00154

| C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|---|---|-----------------------|
| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| A | CHAPLIN DJ et al, Proc. Annu. Meet Am. Assoc. Cancer Res., Mar 1996, Vol.37, #3009, "Antivascular approaches to solid tumour therapy: evaluation of tubulin binding agents". Abstract | 1-15 |
| A | VESZELOVSZKY E et al., European Journal of Cancer, 1993, 29A (3), "Flavone acetic acid and, 5, 6-dimethylxanthenone -4- acetic acid: Relationship between plasma nitrate elevation and the induction of tumour necrosis, pages 404-408 Whole document | 1-15 |